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mRNA Analysis in Reticulocytes of Subjects With Hb D, Hb Porto Alegre, Hb E, and Different Types of Unstable Hemoglobin Variants

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Using a reverse transcription-polymerase chain reaction (RT-PCR) technique we determined the $\alpha2/\alpha1$, α/β, and γ/β mRNA ratios in reticulocytes of 11 patients with seven different unstable β chain variants, of 4 patients with two unstable α chain variants, in hemoglobin (Hb) D, Hb Porto Alegre, and Hb E heterozygotes, and in 8 patients with Hb X-β^{o}-thalassemia (thal) (three D-β^{o}-thal, one Porto Alegre-β^{o}-thal, one Lulu Island-β^{o}-thal, and three E-β^{o}-thal). In addition, we determined the β^{X}/β^{A} mRNA ratios (X = unstable) in some Hb D heterozygotes and in 6 subjects with an unstable β chain variant. Normal α/β and β^{X}/β^{A} mRNA ratios were found in all heterozygotes tested, indicating that the respective mutations did not alter the stability of the mRNAs. The α/β mRNA ratio in four Hb E heterozygotes averaged 4.21 (normal, 4.47), and that in 2 patients with Hb E-β^{o}-thal and four α-globin genes ($\alpha\alpha/\alpha\alpha$) averaged a high 22.4. The γ mRNA level in the Hb E heterozygotes was <1% but varied greatly in patients with Hb E-β^{o}-thal; the $\alpha/(\gamma + \beta)$ mRNA ratios in the 2 patients were 15.5 and 16.7, respectively. The large differences in α/β and $\alpha/(\gamma + \beta)$ mRNA ratios in reticulocytes of subjects with AE and with E-β^{o}-thal may be due to differences in the levels of normally-spliced β^{E} and abnormally-spliced β^{E} mRNAs. Only the latter is unstable and is preferentially produced in bone marrow and reticulocytes of Hb E-β^{o}-thal patients, where it is rapidly degraded.

Key words: RT-PCR, gel electrophoresis, mRNA stability, alternate splicing, mRNA in compound heterozygotes

INTRODUCTION

More than 100 of the nearly 700 known hemoglobin (Hb) variants have decreased stability, presumably because of the types and locations of the amino acid residues that have been replaced, deleted, and/or introduced [1]. Several mechanisms causing Hb instability have been recognized, such as substitutions in the heme pocket or in the interior of the subunit, substitutions that disrupt the secondary structure, deletions of 1–5 amino-acid residues, and certain elongations of the α or β chains, either at the carboxy- or the amino-terminus. An excellent review listing the various unstable variants appeared 5 years ago [2]. The instability of many variants is so severe that a chronic hemolytic anemia results, often with Heinz bodies consisting of mainly α chains or unstable Hb. A few variants exhibit such a greatly increased instability that the abnormal protein cannot be recognized, and their identities are determined through DNA analysis only. Other variants show instability only in in vitro experiments without a clinical hemolysis except for a moderate reticulocytosis.

Although it is generally assumed that the clinical effects depend solely on the presence of the abnormal protein, the possibility that the mutation in the DNA also transcribes into an altered mRNA with decreased stability because of changes in secondary structure of this molecule has not been evaluated. Such a change may result in a decrease in the synthesis of the abnormal message

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TABLE I. Sequences and Locations of Amplification Primers

Primer #	Type	Sequence	Location in β gene
D	Forward	5′-CCTGTGGGGCAAGGTGAA-3′	+92–+108 (exon 1)
738	Forward	5′-AAGGCTCATGGCAAGAAAGTG-3′	+364–+384 (exon 2)
452	Reverse	5′-CCAAAGGACTCAAAGAACCTCTG-3′	+320–+298 (exon 2)
739	Reverse	5′-CCAGCACACAGACCAGCAC-3′	+1376–+1358 (exon 3)
13694	Reverse	5′-TCAAGGCCCTTCATAATATCCCCCA-3′	+1564–+1540 (3′UTR)

Variant	Location of mutation	Primer set	Fragment (bp) cDNA	Fragment (bp) Genomic DNA	Restriction enzyme	Fragment after digestion (bp)[a]	
Yokohama	β31	D + 452	99	229	*Hae*III	β^X, 47	β^A, 58
Bushwick	β74	D + 739	305	1284	*Hae*III	β^X, 264	β^A, 122
Shepherds Bush	β74	D + 739	305	1284	*Hae*III	β^X, 264	β^A, 122
Atlanta	β75	D + 739	305	1284	*Hpa*II	β^X, 184	β^A, 305
Köln	β98	738 + 739	163	1013	*Mae*II	β^X, 143	β^A, 110
D-Los Angeles	β121	D + 13694	493	1473	*Eco*RI	β^X, 493	β^A, 324

[a]Separation on 6% polyacrylamide-7 M urea gel, except for the small Hb Yokohama fragments that were separated on a nondenaturing polyacrylamide gel.

and, thus, in an altered α/β mRNA ratio. We studied this possibility in some 10 mild-to-severe unstable α and β chain variants and included, besides the α/β mRNA ratio, the relative quantities of β^A and β^X (X = unstable) mRNAs in five heterozygotes. Data for normal adults, Hb D, Hb Porto Alegre, and Hb E heterozygotes, as well as compound heterozygotes, are also included.

MATERIALS AND METHODS

Blood samples were collected in vacutainers with EDTA as anticoagulant, and transported in ice to the laboratory or shipped with ice packs by fast air mail service to Augusta, GA. Informed consent was obtained. The subjects studied were, besides normal adults, heterozygotes for one of several (un)stable Hb variants. These were Hb E (from Vietnamese living in Georgia and from Spain); Hb D-Los Angeles (from Safat, Kuwait), including 3 subjects with Hb D-β^O-thalassemia (thal) [3]; Hb D (uncharacterized) and Hb Porto Alegre (from Las Palmas de Gran Canaria, Spain) [4]; Hb Bushwick [5], Hb Köln, and Hb Yokohama [6], all three from Skopje, Macedonia; Hb Shepherds Bush [7] from Philadelphia; Hb Atlanta [8] from Groningen, the Netherlands; Hb Mizuho [9] from s'Hertogenbosch, the Netherlands; Hb Köln and Hb Çapa [10] from Istanbul, Turkey; Hb J-Camagüey [11] and Hb Fannin-Lubbock [12] from Granada, Spain; Hb Lulu Island in combination with β^O-thal [13] from Vancouver, Canada; and Hb E in combination with β^O-thal from Oakland, CA. The amino-acid replacements and corresponding mutations in the genomic DNA are listed in Tables II and III; identification of the abnormalities was made in the authors' laboratory or by the investigator who made the sample available.

Hematological data were obtained with an automated cell counter. Quantitation of the Hb variant or the abnormal α or β chain was by high-performance liquid chromatographic (HPLC) methodology [14,15]. DNA was isolated from white cells by the method of Poncz et al. [16] and, when required, the number of α genes was determined with polymerase chain reaction (PCR)-based methodology [17].

Total RNA was obtained from the blood samples by the method of Chomczynski [18]. Procedures for determining the α2/α1, α/β, and γ/β mRNA ratios have been detailed before [19–21]; determination of α2/α1 and α/β mRNA ratios required one amplification experiment with the five primers A–E added to the Eppendorf tube (details in Fig. 1 of Reference [20]). Six of the 10 mutations resulting in the synthesis of the 10 β chain abnormal Hbs also changed a restriction site in the β-globin gene: a restriction site was created for Hb Atlanta, while such a site was eliminated for the Hbs Yokohama, Bushwick, Shepherds Bush, Köln, and D-Los Angeles. Five mutations occurred in exon 2 (codons 31, 74 (twice), 75, and 98), and one in exon 3 (codon 121). One of the two forward primers used in the experiments was located in exon 1 (#D) and the other in exon 2 (#738), while one of the three reverse primers was in exon 2 (#452), one was in exon 3 (#739), and one was in the 3′ untranslated region (3′UTR) (#13694). Details about the primers are given in Table I; also listed are the primer sets used for the various amplifications, the sizes of the cDNA fragments obtained and those for the corresponding genomic DNAs that would only be observed when the RNA samples were DNA-contaminated, and the sizes of the fragments for β^A and β^X cDNA after digestion with a specific restriction enzyme (− indicates that a restriction site is lost, and + that a site is created by the nucleotide changes specific for the different β chain variants). Figure 1 presents some examples of separations that were obtained; the methodology is the same as described for the determination of

TABLE II. mRNA Ratios in Reticulocytes From Normal Adults and Subjects With Heterozygosity for an Unstable β Chain Abnormal Hb (Average Values Only)

Variant	n	No. of α genes	X^a (%)	F (%)	mRNA α2/α1	α/β	γ/(γ + β)	β^X/β^A
Stable variants								
AA	7	4	0	<0.2	2.63	4.47	0.027	0
AA	3	3	0	<0.2	1.48	3.84	0.017	0
AE (β 26 Glu→Lys; GAG→AAG)	4	4	28.3	<0.2	2.69	4.21	0.003	n.d.
A-D-Los Angeles (β 121 Glu→Gln; GAA→CAA; eliminates *Eco*RI site)	4	4	36.9	<0.2	2.66	4.27	0.018	1.01
AD (undefined)	3	4	46.2	<0.2	2.80	4.16	0.020	n.d.
A-Porto Alegre (β 9 Ser→Cys; TCT→TGT)	5	4	40.8	<0.2	2.59	4.04	n.d.	n.d.
Mildly unstable variants								
A-Bushwick (β 74 Gly→Val; GGC→GTC; eliminates *Hae*III site)	1	4	49.7	0.6	2.47	4.27	0.013	0.92
A-Shepherds Bush (β 74 Gly→Asp; GGC→GAC; eliminates *Hae*III site)	1	4	30.0	<0.2	2.64	4.02	0	0.88
A-Fannin-Lubbock (β 111 Val→Leu; β119 Gly→Asp; GTC→CTC; GGC→GAC)	1	4	41.2	<0.2	2.53	4.42	0.003	n.d.
Moderately unstable variants								
A-Atlanta (β 75 Leu→Pro; CTG→CCG; creates *Hpa*II site)	4	4	46.4	<0.2	2.55	4.29	n.d.	0.99
A-Koln	1	4	n.d.	1.7	2.42	4.02	0.090	0.90
A-Koln (β 98 Val→Met; GTG→ATG; eliminates *Mae*II site)	1	3	n.d.	<0.2	1.30	3.29	0.042	1.06
Severely unstable variants								
A-Yokohama (β 31 Leu→Pro; CTG→CCG; eliminates *Hae*III site)	1	4	7.4	2.6	2.50	4.22	0.090	0.98
A-Mizuho (β 68 Leu→Pro; CTC→CCC)	1	4	0	8.9	2.39	4.54	0.124	n.d.

[a]Hb E contains Hb A_2.

TABLE III. mRNA Ratios in Reticulocytes From Patients With Hb X-β°-Thal

Condition	Sex; age	No. of α genes	Hb F (%)	mRNA α2/α1	α/β	γ/(γ + β)	α/(γ + β)
Hb D-Los Angeles-β°-thal (stable Hb variant) [β121 Glu→Gln; GAA→CAA; codons 37/38 (−T)]							
#615	M; 48 years	3	0.8	1.66	6.79	0.077	6.30
#620	F; 10 years	3	0.6	1.52	6.57	n.d.	
#624	M; 1 year	4	22.5	2.60	10.45	0.436	7.28
Hb Porto Alegre-β°-thal (stable Hb variant) [β9 Ser→Cys; TCT→TGT; codons 8/9 (+G)]							
P.A.	M; 20 years	4	2.8	2.71	7.34	0.046	7.02
Hb Lulu Island-β°-thal (unstable Hb variant) [β107 Gly→Asp; GGC→GAC; codon 15 (TGG→TAG)]							
#8119	F; adult	4	3.2	2.68	9.30	0.060	8.77
Hb E-β°-thal (stable Hb variant)[a]							
#8104	M; 1 year	4	66.8	2.48	25.70	0.659	15.49
#8193	F; 14 years (transfused)	3	1.3	1.33	10.17	n.d.	
#8194	M; 23 years (transfused)	4	11.5	2.70	19.05	0.144	16.65

[a]The β-thal mutations are #8104, IVS-I-5 (G→C); #8193, codon 17 (A→T); #8194, a deletional mutant involving the entire β-globin gene.

relative levels of β^A and β^S mRNAs in Hb S heterozygotes [22].

RESULTS

All data are summarized in Tables II–IV. The β chain abnormal Hbs are divided into three groups according to their instability and the clinical complications associated with the presence of such a variant (Table II). For instance, heterozygosity for Hb Yokohama or Hb Mizuho results in a severe, transfusion-dependent, hemolytic anemia. This is not the case for Hb Atlanta and Hb Köln, although a moderately severe Heinz body hemolytic anemia does exist in these patients. The presence of Hbs Bushwick, Shepherds Bush, and Fannin-Lubbock is clinically less significant, and only a mild hemolytic disease is observed.

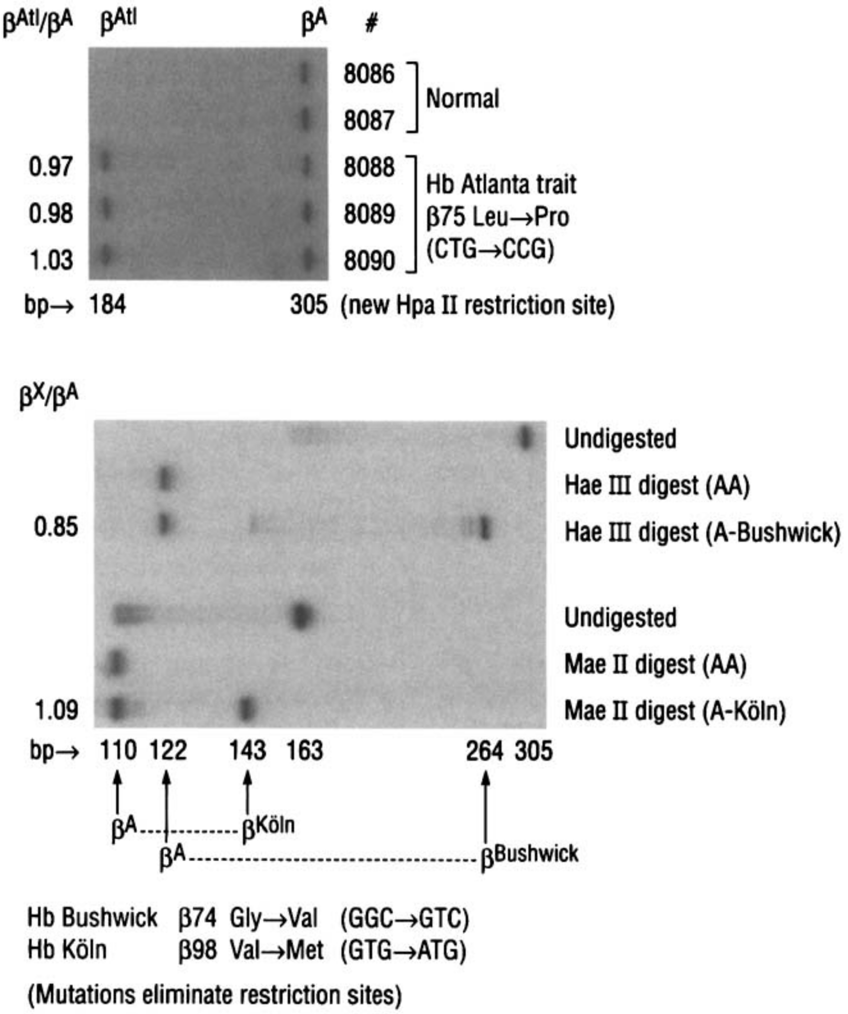

Fig. 1. Separation of ^{32}P-labeled PCR fragments after digestion with the listed restriction enzymes on a 6% denaturing polyacrylamide gel.

Quantities of unstable variants in blood from heterozygotes varied greatly, from 45–50% in patients with Hb Atlanta or with Hb Bushwick, to <1% in the patient with Hb Mizuho. Quantitation of Hb Köln was too inaccurate to provide acceptable values. The mRNA ratios for the 11 patients were compared with those for normal persons, Hb E heterozygotes, Hb D heterozygotes, and Hb Porto Alegre heterozygotes. As 1 of the 2 subjects with Hb Köln carried an α-thal-2 trait, data for normal adults but with three α-globin genes are also included. The normal α2/α1 ratio of 2.63 and α/β ratio of 4.47 are similar to values reported previously [20]; elimination of one of the four α-globin genes decreased the values to 1.48 and 3.84, respectively. Values for the Hb E, Hb D, and Hb Porto Alegre heterozygotes were comparable; individual α/β ratios for the Hb E heterozygotes were 4.37, 4.24, 4.23, and 4.01. The γ mRNA levels in all these individuals were 0–3%. The β mRNA of the four Hb D-Los Angeles heterozygotes consisted of equal amounts of β^A and β^D mRNA; individual ratios were 1.00, 0.94, 1.08, and 1.02. Normal ratios for the α2α1 mRNAs, the α/β mRNAs, and the β^X/β^A mRNAs were also observed for the subjects with an unstable Hb heterozygosity (an exception was the one Hb Köln heterozygote who was also heterozygous for an α-thal-2; his α2/α1 and α/β ratios showed the expected decreases). Increases were seen in the levels of γ mRNA from 1–3% of total γ + β mRNA for the subjects with mildly unstable variants, to 12.5% for the carrier of the Hb Mizuho abnormality.

Table III lists data for 8 subjects with a compound heterozygosity for an abnormal Hb and a β^O-thal. The expected increase in the α/β mRNA ratio was present in the 3 individuals with Hb D-β^O-thal and in the 1 subject with Hb Porto Alegre-β^O-thal; the slightly lower level for the 2 patients with an additional α-thal-2 trait is as expected. A high level of γ mRNA (43.6%) was present in the 1-year-old child, and the α/(γ + β) mRNA ratio was similar to that of an adult β^O-thal heterozygote [3].

TABLE IV. mRNA Ratios in Reticulocytes From Subjects With an Unstable α Chain Variant

Condition	Sex; age	No. of α genes	Hb X (%)	Hb F (%)	mRNA $\alpha2/\alpha1$	mRNA α/β
A-Capa (β94 Asp→Gly; GAC→GGC; in the α1 gene)						
#8188	F; 30 years	4	15.0	<0.2	2.56	4.54
#8189	F; 5 years	4	16.2	0.4	2.68	4.21
#8190	M; 5 years	4	15.6	0.2	2.55	4.76
A-J-Camagüey (α141 Arg→Gly; CGT→GGT)						
#8121	Adult	4	12.1	<0.2	2.87	4.32
#8122	Adult	4	12.0	<0.2	2.89	4.17
AA control						
n = 7		4	0	<0.2	2.53	4.47

The patient with the unstable Hb Lulu Island-β^O-thal with four functional α-globin genes had higher-than-expected α/β and $\alpha/(\gamma + \beta)$ mRNA ratios, suggesting that the β^X (X = Lulu Island) mRNA level was lower than in a simple heterozygote; unfortunately, relatives with Hb Lulu Island heterozygosity only were not available for study. Finally, the data for 2 of the 3 patients with Hb E-β^O-thal showed high α/β ratios (average, 22.4) and high $\gamma/(\gamma + \beta)$ ratios. The calculated $\alpha/(\gamma + \beta)$ averaged 16.1, which is more than twice that observed for the Hb D-Los Angeles-β^O-thal compound heterozygotes. The α/β ratio in the patient with Hb E-β^O-thal and three α-globin genes is, as expected, considerably lower (10.2).

Table IV lists comparable data for two unstable α-globin gene variants; no differences with normal controls were seen in the $\alpha2/\alpha1$ and α/β mRNA ratios.

DISCUSSION

The data from the present study indicate that none of the mRNAs with mutations responsible for the synthesis of the 10 unstable Hb variants have striking instabilities themselves. The normal ratios between the α and β mRNAs and between the β^A and β^X (X = unstable) mRNAs exclude the possibility that an unstable mRNA is a contributing factor to the hematological abnormalities observed in the unstable Hb hemolytic anemia. Even in the most severe cases, the β^x/β^A ratio was nearly one. Admittedly, our present study is limited to a relatively small number of abnormalities, and some other mRNA with a mutation for a different variant might have an altered property. However, the instability of the abnormal globin chain or the Hb type in which it is incorporated appears to be the major cause of hemolytic disease in these subjects. The less stable the abnormal protein, the more severe the condition of the patient. The level of γ mRNA is directly related to severity of disease and the magnitude of persistent anemia. The γ mRNA accounted for 10–13% of the total non-α mRNA in the 2 patients with Hb Yokohama or Hb Mizuho; this level was considerably lower in the other conditions, and often not much different from the values found for the normal adults or the heterozygotes with stable β chain variants.

A few interesting observations were made for the 4 subjects with Hb E trait (Table II) and the 3 patients with Hb E-β^O-thal (Table III). Six subjects had a normal complement of four α-globin genes, and the $\alpha2/\alpha1$ mRNA ratios averaged around 2.5; the Hb E level in the three heterozygotes averaged 28.3% (including Hb A_2). Surprising is the large difference in the average α/β mRNA values; that in the heterozygotes is about the same as in normal adults, suggesting a stable β^E mRNA, but that in the 2 Hb E-β^O-thal patients with four α genes is a high 22–23 (instead of the expected 7–8). Levels of γ mRNA were high, but even the $\alpha/(\gamma + \beta)$ mRNA ratio calculates at least twice the ratio that was expected. Studies in the early 1980's involving β mRNA in bone marrow and reticulocytes of patients with Hb E-β^O-thal or with homozygous Hb E disease [23–26] showed low levels of normally-spliced β^E mRNA in reticulocytes, and a nearly zero level of abnormally-spliced β^E mRNA (due to the alternate splicing site) in the bone marrow. It was concluded that the normally-spliced β^E mRNA was turned over rapidly in the bone marrow. Our results in Hb E-β^O-thal, i.e., the high α/β mRNA ratio in reticulocytes, are in agreement with this conclusion, but the normal α/β ratio in Hb E heterozygotes does not support the concept that normally-spliced β^E mRNA has a decreased stability. On the contrary, this normally-spliced β^E mRNA appears to be present in normal quantities in heterozygotes but not in Hb E-β-thal compound heterozygotes. It may well be that in the latter condition the formation of abnormally-spliced β^E mRNA prevails over that of normally-spliced β^E mRNA; this abnormal mRNA might be present in bone marrow but must be unstable, and is degraded rapidly so that it cannot be detected in reticulocytes. It should be noted that the abnormal β^E mRNA fragment in our experiment is 12 bp shorter than the normally-spliced β^E mRNA fragment, and should be readily detected, but was never observed.

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